Genetic and cytological evidence that heterocyst patterning is regulated by inhibitor gradients that promote activator decay

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The formation of a pattern of differentiated cells from a group of seemingly equivalent, undifferentiated cells is a central paradigm of developmental biology. Several species of filamentous cyanobacteria differentiate nitrogen-fixing heterocysts at regular intervals along unbranched filaments to form a periodic pattern of two distinct cell types. This patterning has been used to exemplify application of the activator-inhibitor model to periodic patterns in biology. The activator-inhibitor model proposes that activators and inhibitors of differentiation diffuse from source cells to form concentration gradients that in turn mediate patterning, but direct visualization of concentration gradients of activators and inhibitors has been difficult. Here we show that the periodic pattern of heterocysts produced by cyanobacteria relies on two inhibitors of heterocyst differentiation, PatS and HetN, in a manner consistent with the predictions of the activator-inhibitor model. Concentration gradients of the activator, HetR, were observed adjacent to heterocysts, the natural source of PatS and HetN, as well as adjacent to vegetative cells that were manipulated to overexpress a gene encoding either of the inhibitors. Gradients of HetR relied on posttranslational decay of HetR. Deletion of both *patS* and *hetN* genes prevented the formation of gradients of HetR, and a derivative of the inhibitors was shown to promote decay of HetR in a concentration-dependent manner. Our results provide strong support for application of the activator-inhibitor model to heterocyst patterning and, more generally, the formation of periodic patterns in biological systems.

activator-inhibitor | *Anabaena* | development

In response to deprivation of fixed nitrogen, the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 differentiates specialized cells called heterocysts, which serve as the site for biological nitrogen fixation of N_2. Heterocysts develop at an interval of approximately every tenth cell along the filament to form a semi-regular pattern of heterocysts from a group of apparently homogeneous vegetative cells. The patterned differentiation of heterocysts is governed in a manner that closely resembles the activator-inhibitor model of biological pattern formation proposed by Geirer and Meinhardt (1–4). This model posits that a self-enhancing activator of differentiation that promotes production of an inhibitor capable of diffusing from source cells and inhibiting activator self-enhancement or promoting activator decay in the neighboring cells is capable of establishing a pattern from a seemingly homogeneous population of cells.

The activator of heterocyst formation, HetR, is a positively autoregulated DNA-binding protein (5, 6). Two inhibitors are encoded by the genes *patS* and *hetN*, both of which are dependent on *hetR* for production and contain the pentapeptide RGSGR, which inhibits heterocyst formation in vivo, and HetR-DNA binding activity in vitro (6–8). *patS*- and *hetN*-dependent inhibitors are synthesized in the developing heterocyst and are thought to diffuse to the neighboring vegetative cells, establishing an inhibitory concentration gradient (7, 8). We report here the observation of a diffusion gradient established by *patS* and *hetN*, via their affect on HetR decay.

Results

Concentration Gradients of HetR Adjacent to Heterocysts. While studying turnover of HetR protein in various genetic backgrounds, we made two observations suggesting that posttranscriptional modulation of HetR-protein levels regulates heterocyst patterning. First, replacement of normal transcriptional control of the chromosomal copy of *hetR* with a copper-inducible promoter (P_{petE}), the activity of which is not developmentally regulated, resulted in heterocyst patterning that was qualitatively similar to that of the wild-type at copper concentrations ranging from 0.3–3 μM (Fig. 1 *A*–*C*). Because levels of transcription from the *petE* promoter are regulated by levels of copper in the medium, expression is similar in all cells for the duration of the experiment. Second, in a strain lacking the *patA* gene, gradients of fluorescence from a HetR-GFP translational fusion under the control of the same inducible promoter were seen adjacent to isolated heterocysts, with fluorescence decreasing with proximity to heterocysts (Fig. 2*A*). Deletion of the *patA* gene from the strain was necessary for visualization of gradients of HetR-GFP fluorescence. Inactivation of *patA* increases the level of HetR in filaments and drastically reduces the number of heterocysts that form, facilitating observation of the effect of single, isolated heterocysts on the levels of HetR-GFP in neighboring cells (9, 10). In contrast, uniform fluorescence was observed using a P_{petE}-*gfp* transcriptional fusion in the same genetic background (Fig. 2*B*). Together, these results suggested that posttranscriptional regulation of HetR-protein levels is determined by proximity to heterocysts and governs final patterning. In all of the work that follows, expression of *hetR* and its derivatives was from the *petE* promoter to avoid the known effects of PatS, HetN, and RGSGR peptide on regulation of transcription from the *hetR* promoter.

To demonstrate that the effect of heterocysts on local HetR-GFP levels is not specific to strains with a Δ*patA* genetic background, alleles of *hetR* known to yield corresponding less active or inactive forms of HetR with reduced turnover rates were used to assess posttranscriptional spatial regulation of HetR in filaments with both a wild-type genetic background and wild-type pattern of heterocysts. *hetR(H69Y)-gfp* and *hetR(S179N)-gfp* (11, 12), which promote the formation of few or no heterocysts, respectively, were introduced into the wild-type strain under the control of the *petE* promoter on a replicative plasmid. In these strains, graded fluorescence similar to that in a Δ*patA* genetic background was observed adjacent to heterocysts in filaments with wild-type het-

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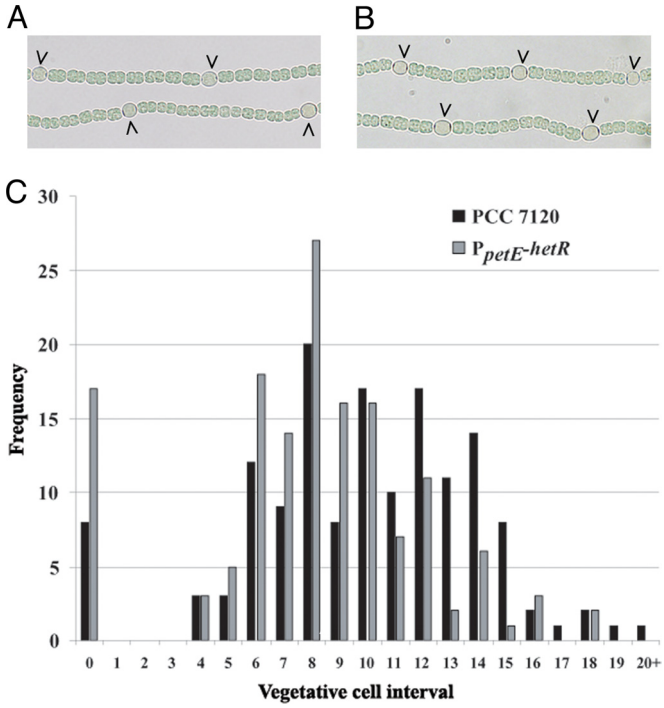

Fig. 1. Heterocyst patterning in a strain with *hetR* under the control of the copper inducible *petE* promoter. Light micrographs of (*A*) *Anabaena* sp. strain PCC 7120 and (*B*) P_{petE}-*hetR*. (*C*) Vegetative cell intervals in the wild-type strain and a strain with *hetR* under the control of the *petE* promoter. Fifty vegetative cell intervals were scored for three replicates 48 h after induction of heterocyst formation by the removal of nitrate and addition of 0.3 μM copper.

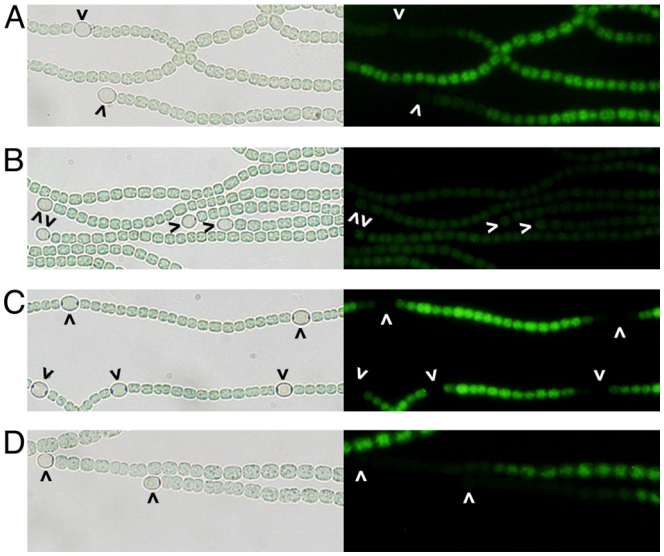

Fig. 2. Observation of a HetR-GFP gradient in proximity to heterocysts. Light (left panel) and fluorescence (right panel) micrographs of: (*A*) Δ*patA*, P_{petE}-*hetR-gfp*; (*B*) Δ*patA*, P_{petE}-*gfp*; (*C*) PCC 7120 with plasmid bearing P_{petE}-*hetR(H69Y)-gfp*; and (*D*) Δ*patA*, P_{petE}-*hetR-gfp* in medium containing nitrate. All micrographs were taken at 48 h after the induction of heterocyst differentiation by the removal of nitrate and addition of 3 μM copper for strains with *hetR* under control of the *petE* promoter, except Fig. 1*D*, which was induced by the addition of 3 μM copper in medium containing nitrate. Carets indicate heterocysts. (Scale bar, 10 μm.)

erocyst patterning, supporting the idea that a signal emanating from heterocysts downregulates levels of HetR in the wild-type organism (Fig. 2*C*). The gradients of fluorescence in this case extended over only 3–4 cells adjacent to heterocysts, compared to 10 or more cells in the case of the *patA* mutant. The difference may be attributable to the increase in HetR levels associated with the alleles of *hetR* on a multicopy plasmid in the former case.

PatS and HetN Cause Posttranslational Decay of HetR. To examine the possibility that the HetR-GFP gradient was established by diffusion of the products of nitrogen fixation from heterocysts to vegetative cells, the *patA*-deletion strain bearing the P_{petE}-*hetR-gfp* fusion was examined in a medium containing both copper and nitrate, a fixed form of nitrogen. Overexpression of HetR in this medium promotes heterocyst formation, but the resulting heterocysts are incapable of nitrogen fixation (13). In the presence of nitrate, a gradient of HetR-GFP was observed in proximity to heterocysts, excluding the possibility that gradients of HetR are established by the products of nitrogen fixation diffusing away from heterocysts (Fig. 2*D*).

Both *patS* and *hetN* are thought to produce diffusible inhibitors of heterocyst formation that act at the level of HetR, and both contain the RGSGR pentapeptide, which is capable of inhibiting heterocyst formation when exogenously added to a culture (7, 8, 14). Therefore, we tested the effect of the addition of RGSGR peptide on the distribution of HetR-GFP. Addition of RGSGR to the *patA*-deletion strain overexpressing HetR-GFP from the *petE* promoter resulted in the condensation of fluorescence to discrete foci within 30 min, and by 3 h, the fluorescence intensity was indistinguishable from background levels (Fig. 3*A*). Addition of RGSGR to a culture overexpressing GFP alone had no affect on the level or distribution of fluorescence (Fig. S1). Western blot analysis showed that levels of wild-type and GFP-tagged HetR decreased over time in response to RGSGR peptide, consistent with the decrease in fluorescence observed for the GFP-tagged HetR (Fig. 3*B*). The addition of RGSGR at concentrations between 0.1–1 μM resulted in a graded reduction in HetR levels in a *patA*-deletion strain, and addition of 1 μM or more of RGSGR resulted in a decrease in HetR levels in a wild-type background (Fig. 3*C*). To determine if the decrease in HetR levels was the result of posttranslational regulation of HetR protein, we inhibited translation with the aminoglycoside antibiotics spectinomycin and streptomycin before addition of RGSGR. In the absence of new protein production, addition of RGSGR peptide resulted in a substantial decrease in HetR compared to background HetR turnover, demonstrating that the effect on HetR is posttranslational (Fig. 3*D*). The effect of RGSGR on HetR levels was the same regardless of the nitrogen source used for growth of filaments.

To determine if *patS* and *hetN* affect HetR turnover in vivo, *hetR-gfp* was overexpressed along with either *patS* or *hetN* in a *patA*-deletion or wild-type strain. Overexpression of either *patS* or *hetN* resulted in a decrease in HetR levels consistent with those seen upon addition of RGSGR (Fig. 4*A*). A mutant allele of *hetN*, *hetN(RGDAR)*, that substitutes two residues in the RGSGR motif was incapable of promoting HetR turnover or inhibiting heterocyst formation, suggesting that the RGSGR motif of HetN is necessary for the activity of HetN on HetR levels and heterocyst inhibition.

Levels of HetR are known to accumulate in strains lacking HetF, a predicted protease (10) and in strains with *hetR(S179N)*, which codes for a version of HetR lacking reported autoproteolytic activity (10, 15). To test if PatS- and HetN-dependent turnover of HetR requires HetR autoproteolysis or HetF, *patS* or *hetN* was overexpressed in a *hetF*-mutant strain as well as in a strain with *hetR* replaced with *hetR(S179N)*, and levels of HetR or HetR(S179N) were detected by immunoblotting. PatS and HetN reduced levels of HetR in strains lacking HetF or with

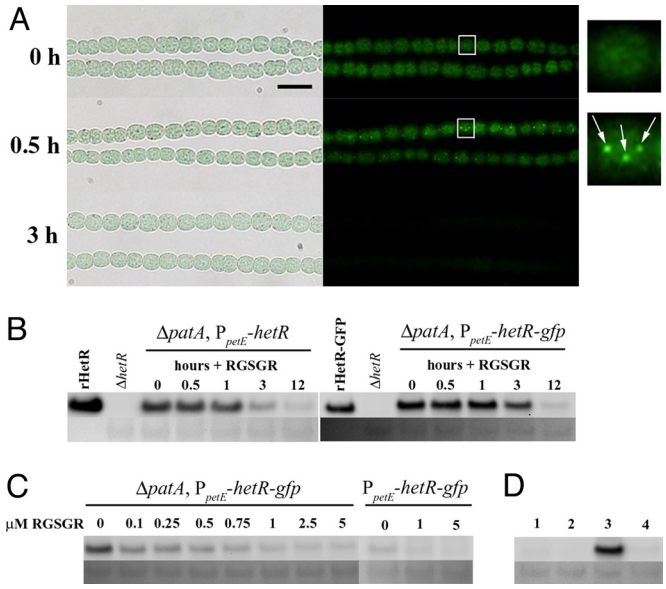

Fig. 3. RGSGR pentapeptide promotes degradation of HetR. (*A*) Light (left panel) and fluorescence (right panel) micrographs of Δ*patA*, P_{petE}-*hetR-gfp* at various times (as indicated) after the addition of RGSGR (2 μM). Panels to the far right are at magnification 4× fluorescence micrographs as indicated by white squares, and arrows indicate HetR-GFP foci. (Scale bar, 10 μm.) (*B*) Western blot analysis of wild-type and GFP-tagged HetR from strains with the indicated genetic backgrounds at the times indicated after addition of RGSGR (2 μM). (*C*) Western blot analysis of HetR levels from strains with the indicated genetic backgrounds in response to varying concentrations of RGSGR. (*D*) Inhibition of translation before addition of RGSGR: (Lane 1) Δ*patA*, P_{petE}-*hetR-gfp* without copper induction; (lane 2) incubation with streptomycin and spectinomycin (5 μg/mL) for 3 h followed by addition of copper for 24 h; (lane 3) addition of copper for 24 h followed by addition of streptomycin and spectinomycin for 15 h; (lane 4) addition of copper for 24 h followed by addition of streptomycin and spectinomycin for 3 h followed by addition of RGSGR for 12 h. All samples were cultured in medium containing nitrate and 3 μM copper for induction of *hetR* except where indicated otherwise. For Western blot analysis, 8 μg total cellular protein were loaded; upper panel is Western blot with anti-HetR polyclonal antibodies, and lower panel is a Coommassie-stained section of the membrane for equality of protein loading.

hetR(S179) replacing the wild-type copy of *hetR* (Fig. S2), indicating that PatS- and HetN-dependent turnover of HetR does not require HetF- or HetR-protease activity.

Overexpression of *patS or hetN* Is Sufficient for Recreation of Gradients of HetR. To determine if expression of *patS* or *hetN* from only a small group of cells in filaments incapable of forming heterocysts is sufficient for the creation of gradients in neighboring cells, genetic mosaic filaments (10) were constructed by introducing a plasmid expressing both *gfp* and *patS* or *hetN* into small groups of cells of a strain with P_{petE}-*hetR(S179N)-gfp* in place of the normal chromosomal copy of *hetR*. In *Anabeana*, high levels of autofluorescence limit the emission wavelengths that are available for reporter proteins, so GFP fluorescence was used to track both HetR levels and cells that were overexpressing *patS* or *hetN*. The two sources of fluorescence were distinguished by the formation of fluorescent foci after the addition of RGSGR, which is observed only with HetR-GFP. Use of the *hetR(S179N)* allele insured that the results would not be complicated by the possibility of pre-established gradients near the sites of heterocyst formation, because this allele is incapable of promoting heterocyst formation (11). In the genetic mosaic filaments described here, uniform GFP fluorescence indicates cells that are overexpressing *patS* or *hetN* from a plasmid. These cells would be the only ones expressing either gene at a significant

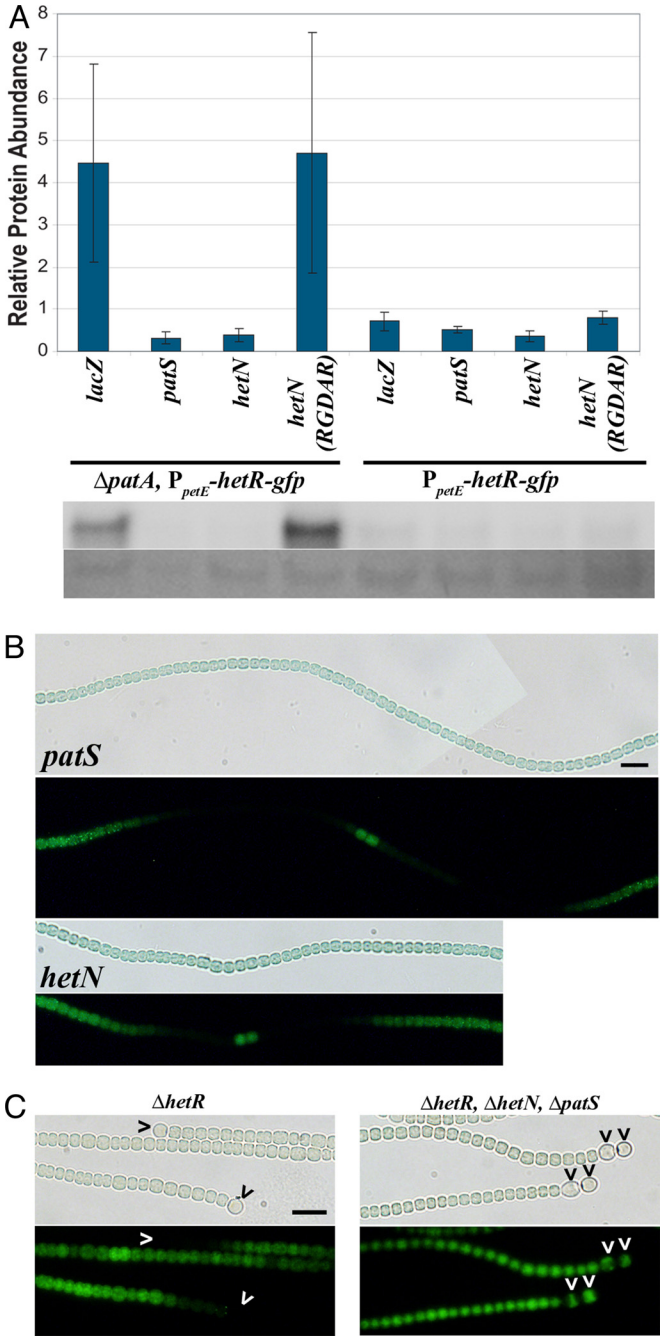

Fig. 4. *patS* and *hetN* promote degradation of HetR and formation of HetR-GFP gradients. (*A*) Protein quantification (error bars ± SD, $n = 3$) and representative Western blot (as described in Fig. 3) of HetR in strains overexpressing *lacZ*, *patS*, *hetN*, or *hetN(RGDAR)*. (*B*) Light (upper panel) and fluorescence (lower panel) micrographs of mosaic filaments of P_{petE}-*hetR(S179N)-gfp* with plasmid bearing P_{nir}-*gfp* and P_{petE}-*patS*, or P_{petE}-*hetN* (as indicated) 72 h after introduction of plasmid, 10 min after addition of RGSGR (20 μM). (Scale bar, 10 μm.) (*C*) Light (upper panel) and fluorescence (lower panel) micrographs of a *hetR*-deletion strain, or *hetR*-, *patS*-, *hetN*-deletion strain (as indicated) with plasmid bearing P_{petE}-*hetR(H69Y)-gfp* 48 h after induction of heterocysts by removal of nitrate and addition of 3 μM copper. Carets indicate heterocysts. (Scale bar, 10 μm.)

level, because the strain lacks a functional copy of *hetR*, which is necessary for induction of expression of each from its native promoter. On the other hand, fluorescence levels from cells with discreet fluorescent foci is indicative of HetR(S179N)-GFP

levels. In experiments with genetic mosaic filaments, groups of cells with uniform fluorescence expressing GFP from the plasmid were flanked by cells with levels of fluorescence that increased with distance from cells overexpressing *patS* or *hetN* (Fig. 4*B*). Graded areas of fluorescence consistently formed discrete foci upon addition of RGSGR peptide, while fluorescence in cells between two graded areas remained uniform, demonstrating that areas of graded fluorescence represent HetR-GFP. Thus, expression of either *patS* or *hetN* is sufficient for establishment of concentration gradients of HetR along filaments. These results also conclusively demonstrate that PatS- and HetN-dependent signals produced in one cell are capable of affecting levels of HetR over a distance of several cell lengths.

PatS or HetN Is Necessary for Gradients of HetR Adjacent to Heterocysts. To test whether *hetN* and *patS* are solely responsible for formation of concentration gradients of HetR in proximity to heterocysts, both were deleted from the chromosome, and a form of HetR with severely reduced activity was used to report levels of HetR. Because wild-type *hetR* in a strain lacking both *patS* and *hetN* results in nearly complete differentiation of vegetative cells to heterocysts (16), we used the *hetR(H69Y)* allele of *hetR*, which promotes formation of rare heterocysts, even in the absence of *patS* and *hetN*. In a *hetR*-deletion strain with a plasmid bearing *hetR(H69Y)-gfp* under the control of the *petE* promoter, graded fluorescence was observed adjacent to heterocysts (Fig. 4*C*). Concentration gradients of HetR were still observed in strains with either *patS* or *hetN* individually deleted. However, deletion of both genes completely abolished the HetR gradient, consistent with *patS* and *hetN* being exclusively and independently responsible for establishing concentration gradients of HetR (Fig. 4*C*).

Discussion

There is no evidence for the intercellular diffusion of HetR, a cytoplasmic protein of 299 aa, and our work with genetic mosaics has shown that there is no increased tendency for differentiation of cells adjacent to those overexpressing *hetR*. Because both PatS and HetN contain the RGSGR peptide motif and Golden's group has shown that confinement of PatS to source cells prevents proper heterocyst patterning (7), it seems likely that portions of PatS and HetN that include the RGSGR motif constitute diffusible inhibitory signals. The foci of HetR-GFP fluorescence elicited by RGSGR peptide are strikingly similar to those recently observed when various Clp proteases and their substrates were fused to GFP in *Bacillus subtilis* and *Caulobacter crescentus* (17–19), suggesting that PatS- and HetN-dependent signals may target HetR for transport to, and degradation by, cellular proteases located at discreet positions in the cell. PatS- and HetN-dependent decay of HetR is distinct from previously reported HetR-autoproteolysis or HetF-dependent degradation as evidenced by decay of $HetR_{S179N}$, which is not autoproteolytic (15), and decay of HetR in a *hetF*-mutant background. We propose that diffusion gradients of PatS- and HetN-derived signals mediate the formation of inverse gradients of HetR by targeting HetR for degradation by an unknown protease or proteases (Fig. 5).

HetN is not necessary for formation of the initial pattern of heterocysts that forms in response to limiting fixed nitrogen in the environment, but, instead, it regulates maintenance of heterocyst patterning thereafter as the filament elongates by cellular growth and division (8). On the other hand, PatS is clearly involved in initial pattern formation and plays a less substantial role in its maintenance (7, 20). Our results suggest that the mechanics of pattern formation and maintenance are remarkably similar, both involving concentration gradients of HetR. Concentrations of HetR increase with increasing distance from cells producing significant amounts of PatS or HetN.

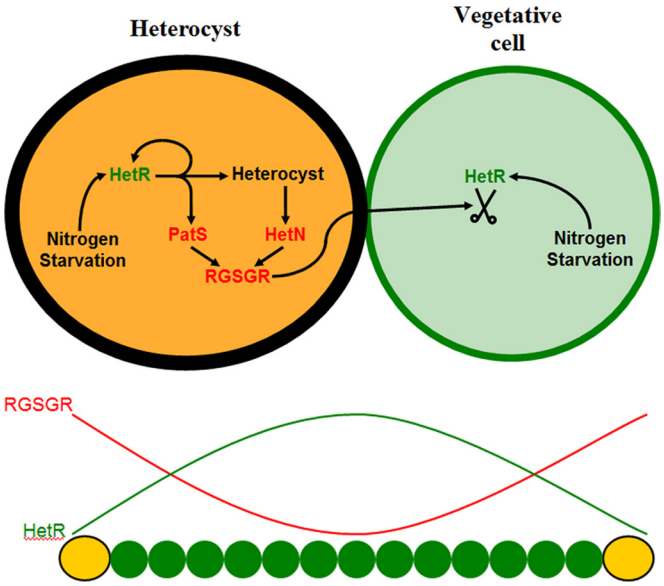

Fig. 5. A schematic diagram of the molecular regulation of heterocyst patterning. The activator of heterocyst formation, HetR, is expressed in all cells in response to a lack of fixed nitrogen. HetR is positively autoregulated and directly or indirectly promotes other factors leading to the development of heterocysts as well as the inhibitors PatS and HetN. The active portion of PatS and HetN contains the RGSGR pentapeptide, which promotes HetR decay. A portion of PatS and HetN containing the RGSGR pentapeptide diffuses away from source cells to neighboring cells establishing an inhibitor concentration gradient along the filament, which in turn promotes an inverse gradient of the activator, HetR. Arrows indicate positive regulation. Scissors indicate decay.

Expression of *patS* is induced early during initial pattern formation, but returns to a basal level after morphological differentiation is complete. This is consistent with our observation that gradients of HetR were observed adjacent to only about 50% of heterocysts in the Δ*hetN* background after the strain had differentiated multiple rounds of heterocysts. Presumably, the "older" heterocysts had ceased expression of *patS*. Conversely, gradients of HetR were always observed adjacent to heterocysts in the Δ*patS* background regardless of the time after induction of differentiation, presumably because production of HetN continues indefinitely in mature heterocysts.

The activator-inhibitor model of biological pattern formation has been applied to a wide range of developmental systems including those that generate periodic patterns, such as trichome formation on plant leaves, mammalian hair patterning, and feather branching in birds (21–23). It predicts the formation of concentration gradients of activator and inhibitor (1). Here we have demonstrated the formation of concentration gradients of activator in the cyanobacterium *Anabaena* in response to two inhibitors of differentiation. Direct observation of diffusion of the inhibitors, however, remains elusive. The model also stipulates that the inhibitor limit positive autoregulation of the activator or promote its decay. The RGSGR pentapeptide prevents binding of HetR just upstream of the transcriptional start point implicated in positive autoregulation of HetR (6), suggesting that the inhibitors PatS and HetN promote decay of HetR and limit positive autoregulation at the point of transcription. However, induction of transcription of *hetR* in response to deprivation of combined nitrogen does not occur in a spatially graded manner, probably as a consequence of the involvement of other regulatory proteins in transcriptional autoregulation (10). It will be interesting to see if other periodic biological patterns that conform to the activator-inhibitor model share

some of the general molecular interactions that govern formation and maintenance of heterocyst patterning.

Materials and Methods

The growth of *Escherichia coli* and *Anabaena* sp. strain PCC 7120 and its derivatives; concentrations of antibiotics; the regulation of P_{petE} and P_{nir} expression; conditions for photomicroscopy; conjugal transfer of plasmids from *E. coli* to *Anabaena* sp. strain PCC 7120 and its derivatives; construction of mosaic filaments; and Western blot analysis were performed as previously described (10, 12, 24). To generate rHetR-GFP, pDR292 was introduced into *E. coli* strain BL21 (DE3). Quantification of relative protein abundance of HetR-GFP was determined via Western blot analysis by generating a standard curve of rHetR-GFP based on serial dilution of BL21 (DE3) carrying plasmid pDR292, running these standards with experimental data and detecting and quantifying the signal using the GeneGnome BioImaging System and GeneTools Software (Syngene).

Plasmid and Strain Construction. The plasmids, strains, and primers used in this study are listed in Tables S1 and S2, and their construction is described in *SI Text*.

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